Iron metabolism in patients with Graves' hyperthyroidism

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Abstract: OBJECTIVES: Graves' hyperthyroidism (GH) interferes with iron metabolism and elevates ferritin. The precise mechanisms remain unclear. The influence of thyroid hormones on the synthesis/regulation of hepcidin, an important regulator of iron metabolism, remains uncharacterized. DESIGN: Prospective observational study. PATIENTS: We included patients (n = 31) with new-onset and untreated GH. MEASUREMENTS: Laboratory parameters indicative of iron metabolism (ferritin, transferrin, hepcidin), inflammatory markers/cytokines and smoking status were assessed at the diagnosis of GH (T0) and at euthyroidism (T1) in the same patients using multivariable analyses. Hepcidin was measured by mass spectrometry (hepcidinMS) and ELISA (hepcidinEL). The impact of T3 on hepatic hepcidin expression was studied in a cell culture model using HepG2 cells. RESULTS: Median ferritin levels were significantly lower and transferrin significantly higher at T1 than at T0. HepcidinMS levels were lower in males and females at T1 (statistically significant in males only). No statistically significant difference in hepcidinEL was detected between T0 and T1. Plasma levels of inflammatory markers (high-sensitive CRP, procalcitonin) and cytokines (interleukin 6, interleukin 1ß, tumour necrosis factor) were not different between T0 and T1. Smokers tended to have lower fT3 and fT4 at T0 than nonsmoking GH patients. T3 significantly induced hepcidin mRNA expression in HepG2 cells. CONCLUSIONS: Iron metabolism in patients with GH undergoes dynamic changes in patients with GH that resemble an acute-phase reaction. Inflammatory parameters and cytokines were unaffected by thyroid status. Gender and smoking status had an impact on ferritin, hepcidin and thyroid hormones.

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Abstract

Objectives:

Graves' hyperthyroidism (GH) interferes with iron metabolism and elevates ferritin. The precise mechanisms remain unclear. The influence of thyroid hormones on the synthesis/regulation of hepcidin, an important regulator of iron metabolism, remains uncharacterized.

Design:

Prospective observational study.

Patients:

We included patients (n=31) with new-onset and untreated GH.

Measurements:

Laboratory parameters indicative of iron metabolism (ferritin, transferrin, hepcidin), inflammatory markers/cytokines and smoking status were assessed at the diagnosis of GH (T0) and at euthyroidism (T1) in the same patients using multivariable analyses. Hepcidin was measured by mass spectrometry ($hepcidin_{MS}$) and ELISA ($hepcidin_{EL}$). The impact of T3 on hepatic hepcidin expression was studied in a cell culture model using HepG2 cells.

Results:

Median ferritin levels were significantly lower and transferrin significantly higher at T1 than at T0. $Hepcidin_{MS}$ levels were lower in males and females at T1 (statistically significant in males only). No statistically significant difference in $hepcidin_{EL}$ was detected between T0 and T1. Plasma levels of inflammatory markers (high-sensitive CRP, procalcitonin) and cytokines (interleukin 6, interleukin 1ß, tumor necrosis factor α) were not different between T0 and T1. Smokers tended to have lower fT3 and fT4 at T0 than non-smoking GH patients. T3 significantly induced hepcidin mRNA expression in HepG2 cells.

Conclusions:

Iron metabolism in patients with GH undergoes dynamic changes in patients with GH that resemble an acute phase reaction. Inflammatory parameters and cytokines were unaffected by thyroid status. Gender and smoking status had an impact on ferritin, hepcidin and thyroid hormones.

Introduction

Hyperthyroidism is a systemic disorder. Apart from well-known implications of thyroid overfunction for example on the cardiovascular system and bone metabolism, thyroid hormone excess interferes also with iron metabolism, i.e. elevates ferritin and has an impact on other hematologic parameters. These abnormalities are temporary and normalize once the patient has achieved an euthyroid state. In severe hyperthyroidism normochromic, normocytic anemia may be associated with impaired iron use[1, 2]. These anemias are unresponsive to hematinic therapy[2] (i.e. substitution with iron, vitamin B12 or folate) and vanish once normal thyroid function is restored. On the other hand, there are manifold effects of thyroid hormones on iron metabolism.

Iron acts as an important cofactor in fundamental biochemical processes, for example oxygen transport. High iron concentrations are toxic and therefore iron metabolism in the body is highly regulated. Several proteins play a critical role in iron metabolism. Iron, bound to *transferrin*, is transported within the plasma and internalized by receptor-mediated endocytosis. Intracellular *ferritin* is principally responsible for storage and buffering of excess iron[3]. *Hepcidin*, a liver derived peptide-hormone, is one of the most important regulators of iron homeostasis. It lowers plasma iron by down regulating ferroportin 1 (FPN-1), which promotes iron efflux out of cells (enterocytes, hepatocytes or macrophages)[4, 5]. The bioactive form of hepcidin (hepcidin-25) contains 25 amino acids. Smaller isoforms (hepcidin-24, -23, -22) are usually present in conditions characterized by elevated hepcidin-25 levels; however, the biological significance of these isoforms remains unclear[6-8].

Acute-phase reactions lead to substantial changes in iron metabolism and are characterized by high levels of ferritin and hepcidin and low levels of iron and transferrin[3]. Animal models of hyperthyroidism demonstrate decreased circulating plasma iron and increased hepatic iron and ferritin. In addition, hepatic ferritin synthesis is increased, although hepatic albumin synthesis remains unchanged[9]. In hyperthyroid states plasma erythropoietin (EPO) levels are high, erythropoiesis is increased, and bone marrow is hypercellular[2, 10, 11]. Hence, iron turnover due to augmented iron utilization is increased, but iron overload is absent[2, 10, 12], and the concomitant expansion of plasma

volume masks the increase in red blood cell mass[12]. The observed decline in plasma iron levels can be explained by faster incorporation of iron into heme; however, any impact of hepcidin remains speculative because there is no data regarding the influence of thyroid hormones on hepcidin synthesis and regulation. Case reports and case series have described transiently elevated ferritin levels in hyperthyroid patients that decreased after achieving a euthyroid state[13–16]. Whereas some studies found a positive correlation between levels of thyroid hormones and ferritin[15], others did not[14]. Furthermore, the mechanisms of hyperthyroidism-associated hyperferritinemia remain poorly understood.

Here, we hypothesize that thyroid hormones directly influence iron metabolism by affecting ferritin and/or hepcidin levels. Therefore, the *aims* of this study were:

a) To quantify the levels of ferritin, hepcidin, and other parameters related to iron metabolism (e.g. transferrin) in patients with Graves' hyperthyroidism (GH) and in the same patients after they had achieved euthyroidism.
b) To investigate potential mechanisms by measuring inflammatory parameters and cytokines and to set these measurements in relation to gender and smoking status.
c) To study the impact of thyroid hormones on hepatic hepcidin expression using a cell culture model.

Subjects and Methods

This was a prospective observational study. The subjects consisted of patients who attended our outpatient clinic with newly diagnosed and untreated Graves' hyperthyroidism (GH). Exclusion criteria were: age below 18 years, pregnancy/planned pregnancy, known hepatopathy, alcohol abuse (defined as consumption of ≥3 standard drinks per day in females or ≥4 standard drinks per day in males), known/treated iron deficiency, active malignancy, chronic renal insufficiency (≥ chronic kidney disease stage III) and acute/chronic infections. Laboratory parameters were assessed in patients at the diagnosis of GH (T0) and after they reached euthyroid function (T1) following treatment with antithyroid therapy (carbimazole or propylthiouracil). Euthyroid function was defined as the presence

of normal thyroid stimulating hormone (TSH)/free triiodothyronine (fT3) and free thyroxine (fT4) values for at least four weeks. Thyroid volume was calculated using B-mode standard ultrasonography. All patients gave their written informed consent. The study conformed to the declaration of Helsinki and was approved by the local ethics committee (approval number: EKNZ-13077).

Laboratory assessment

Indicators of thyroid (TSH, fT3, fT4, anti-TSH-receptor antibodies) function, iron metabolism (ferritin, transferrin, iron, soluble transferrin receptor [sTfR]), and inflammation (high sensitivity c-reactive protein [hsCRP], procalcitonin) were measured using electrochemiluminescence immunoassays (ECLIA, Roche COBAS® System, Roche Diagnostics, Rotkreuz, Switzerland). Ferritin index was calculated using the quotient of sTfR / log ferritin.

Aliquots were kept at -70 °C for batch analysis of hepcidin and cytokines. Hepcidin analyses were performed using two different assays: mass spectrometry for the determination of bioactive hepcidin-25 ($hepcidin_{MS}$), which represents the most specific and accurate approach for detecting bioactive hepcidin[6, 8, 17]. For comparison, hepcidin was also measured by ELISA ($hepcidin_{EL}$, Hepc Cusabio®, College Park MD, USA), which detects hepcidin-25 and other hepcidin-isoforms. Measurement of cytokines was performed using a flowcytometric method.

Cell culture

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (1g/L glucose) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For gene expression analysis, 2×105 cells/well were seeded into 6-well plates. After attachment, cells were washed in phosphate buffered saline (PBS) and incubated in serum-free DMEM for 24 h. Thereafter, cells were stimulated with ± 10 nM T3 (Sigma-Aldrich, Buchs, Switzerland) for 24 h in serum-free DMEM. After washing with PBS, RNA was extracted (NucleoSpin®, Macherey-Nagel, Düren, Germany), reverse transcribed (Takara Bio Europe, Saint-Germain-en-Laye, France), and quantitative real-time

PCR was performed as described[18]. Hepcidin primers (Hs00221783_m1) were purchased from Applied Biosystems (ThermoFisher Scientific, Waltham MA, USA).

Statistical analysis

The values for each parameter were compared at baseline (T0) and follow-up (T1), as defined above. Initially, data were analyzed descriptively by calculating medians and interquartile ranges (IQRs). The statistical significance of any differences was established using the non-parametric Wilcoxon matched-pairs signed-rank test. For the main variables (fT3, fT4, ferritin, hepcidin and transferrin), separate multivariable analyses were performed using random effects models with a random intercept. Standard errors were estimated with the Huber-White sandwich method[19] because of observed variance heteroscedasticity. Only patients without missed measurements at either T0 or T1 were included in the regression analysis. Analyses were repeated by including also patients with partially missing data (hepcidin could not be measured in 6 patients at T0, 1 patient had no measurements of cytokines at T0), with no alteration of the results or conclusions.

The main exposure of interest in our multivariable analysis was the time-point (baseline T0 or follow-up T1). The following variables were considered for model adjustment: age, gender, smoking status, and time between baseline and follow-up (in days). We also tested for interactions with gender and smoking status. These interaction tests were specified *a priori* and were based on existing evidence in the literature. The variables were manually added to the model in an incremental fashion and the results were inspected visually. The final models only included those adjustments that exerted a substantial impact on the point estimate for the time-point (>20%) and interaction terms with $P < 0.1$. All data analyses were performed using Stata SE Version 13.1 (StataCorp LLC, College Station TX, USA). Statistical significance was defined as $P < 0.05$. All tests were two-sided. No adjustments for multiple testing were performed.

Results

Thirty-one patients (22 females, 9 males; mean age 48 years [range 26–82]) were included in the study. Twelve out of thirty-one (38%) patients were smokers. The median (IQR) thyroid volume was 13.9 (10.6–23.1) ml, thyroid parameters are displayed in **Table 1**. Patients were treated either with carbimazole (n=29) or propylthiouracil (n=2). Two patients had normochromic, normocytic anemia at baseline that resolved at T1, but none had laboratory values consistent with iron deficiency (i.e. ferritin index ≥3 or soluble transferrin receptor >5 mg/l).

Restoring normal thyroid function led to a rise of *transferrin* and *plasma iron* (values for iron at T1 not significant). By contrast, transferrin saturation and soluble transferrin receptor levels were unaffected by thyroid function as were plasma levels of *inflammatory markers* (hsCRP and procalcitonin) and *cytokines* (IL-6, IL-1ß, and TNF-α, **Table 1**). Smoking status had no impact on levels of inflammatory markers and cytokines (data not shown).

Median (IQR) *ferritin* concentrations at baseline were 156 (63–262) µg/l and were higher in males (277 [222.5–615] µg/l) than in females (117.5 [56.75–210] µg/l). The highest ferritin values were 1100 µg/l in one male and 443 µg/l in one female patient at T0, but in both of these patients the values had normalized at T1. Median ferritin levels decreased significantly after euthyroid function had been achieved (**Table 1, Fig. 1**).

Median (IQR) *hepcidin$_{MS}$* concentrations at baseline were 9.15 (6.45–17.25) nmol/l and were higher in males (17.1 [10.5–26.9] nmol/l) than in females (8.3 [6.3–13.6] nmol/l). As with ferritin, hepcidin$_{MS}$ levels decreased in males and females after patients had reached euthyroidism. This change was only statistically significant in men (**Table 1, Fig. 2**). Changes between T0 and T1 did not reach statistical significance when hepcidin was measured by ELISA (hepcidin$_{EL}$, **Table 1**). There was no correlation detected between Anti-TSH receptor antibody (TRAb) titers and ferritin/hepcidin levels (data not shown).

Multivariable regression analyses

The following four parameters were also subjected to multivariable regression analyses: ferritin, $hepcidin_{MS}$, fT3, and fT4 (**Table 2**). While the patterns of change observed in univariable analyses were largely replicated, multivariable analyses also indicated confounding by age (ferritin) and, more importantly, effect modification by smoking status and sex. With respect to the latter, the changes in ferritin and hepcidin differed significantly between males and females, with males having higher values at baseline but a subsequent steeper decline. At follow-up, the differences were still statistically significant between the two sexes for ferritin (estimated difference between males and females [95% confidence interval (CI)]: 103.9 µg/l [22.9; 184.9]), but not for hepcidin (2.5 nmol/l [-0.9; 6.0]).

Further, the regression models for fT4 and fT3 indicated differences in the dynamics of changes between smokers and non-smokers (**Fig. 3a/3b**). In particular, smokers had lower fT3 and fT4 at T0 (model estimates [95% CI] for differences between non-smokers and smokers of -6.1 pmol/l [-11.1; -1.2] and -18.2 pmol/l [-31.1; -5.2], respectively). During follow-up, these laboratory parameters stabilized at similar levels for smokers and non-smokers (differences of 0.3 pmol/l [-0.2; 0.9] and-0.1 pmol/l [-2.2; 2.0], respectively.

Cell culture model

To investigate whether elevated hepcidin levels in hyperthyroid subjects might be directly driven by high fT3 levels, experiments were performed in a human liver cell line. As shown in **Fig. 4**, T3 treatment significantly induced hepcidin mRNA expression in HepG2 cells, consistent with the findings in patients.

Discussion

The results of our study are threefold: **a)** GH leads to changes in iron metabolism that resemble a classical "acute phase reaction" i.e. elevation of ferritin and hepcidin and a decline of transferrin and iron levels. These changes seem to be (in part) independent from inflammatory parameters and normalize after achieving euthyroid function. **b)** There is an impact of gender and smoking status on these parameters, i.e. ferritin and hepcidin levels are influenced by gender, fT3 and fT4 by smoking status. **c)** Thyroid hormones have a direct impact on hepcidin mRNA expression in the cell culture model.

Regulation of the synthesis and secretion of ferritin and hepcidin is complex, and involves multiple pathways and effectors, including total iron content, reactive oxygen species, erythropoietin (EPO) levels, and cytokines[20]. During acute-phase reactions, a variety of cytokines and proinflammatory molecules (TNF-α, IL-1α, IL-1β, IL-6, lipopolysaccharide and nitric oxide) regulate ferritin by inducing the transcription, stimulating the secretion, and modulating the posttranscriptional mechanisms[21]. IL-6 is one of the primary inducers of hepcidin[22, 23] in inflammatory and infectious states. In contrast, EPO suppresses hepcidin levels[24], however, the effect of the high EPO levels in hyperthyroid patients[10] on hepcidin synthesis may be overridden by other factors (e.g. thyroid hormones).

New-onset GH can be interpreted as a form of acute-phase or inflammatory reaction, but markers of inflammation and inflammatory cytokines in our patients were not affected neither by thyroid function nor by smoking status. These findings are in concordance with other studies where circulating serum cytokines are unchanged in GH patients[25] or only partially elevated[26].

Therefore, a direct hormonal influence on the physiology of ferritin and hepcidin must be assumed. Cell culture models indeed demonstrated that ferritin gene transcription was induced by insulin/IGF-1[27], TSH[28] and T3[29], and that the latter modulates posttranscriptional mechanisms via iron regulatory proteins (IRPs)[29]. This is the first study to show a direct effect of T3 on hepcidin mRNA expression in

a human liver cell line, which is consistent with the clinical finding of elevated hepcidin levels in our hyperthyroid subjects. Of note, the impact of T3-stimulation on hepcidin production in HepG2 cells was assessed on mRNA but not on protein levels, as hepcidin production may mainly be regulated at transcriptional level[30].

In our study population, ferritin and hepcidin levels showed wide variability and were higher in men than in women. The influence of gender on ferritin[31] and hepcidin[32] is well known. Estrogen inhibits hepcidin synthesis[33] and hepcidin levels are lower in premenopausal subjects[32] and in women with high endogenous estrogen levels resulting from *in vitro* fertilization procedures[34]. In our study, the majority (16/22) of the female patients were premenopausal and thus had lower hepcidin levels, which may have contributed to the difficulty in detecting differences in hepcidin levels between T0 and T1 in female subjects.

Compared to smokers, non-smokers had significantly higher fT3 and fT4 values at T0. Smoking has an impact on various aspects of thyroid function and the development of thyroid disease[35]. Smokers are exposed to higher thiocyanate levels[36], which can affect T3 and T4 concentrations[37], mainly through its inhibitory effect on iodine trapping[38]. In addition, lower T3 and T4 levels have been described in smokers with normal thyroid function[39, 40]. However, to the best of our knowledge, the impact of smoking on T3 and T4 levels in patients with hyperthyroidism and GH has not been described until now.

In comparison to other studies[13–16], this study had the advantages of including only patients with GH, using longitudinal assessments of laboratory values, and measuring a novel variable related to iron metabolism (hepcidin). Our study design allowed us to compare and demonstrate the dynamic changes in ferritin and hepcidin that occur in the same individuals during thyroid hyperfunction and after the achievement of normal thyroid function. This approach allowed the elimination of high inter-individual variability in ferritin and hepcidin levels.

Another strength of our study was the use of mass spectrometry, the most accurate method for the quantification of bioactive hepcidin. A recently published study[34] found no differences in circulating hepcidin levels in 20 patients with hyperthyroidism (17 with GH, two with subacute thyroiditis and one with toxic nodular goiter). However, hepcidin levels in that study were measured by ELISA only, which has the drawback of low specificity and accuracy. This is in line with our findings showing no statistically significant differences in hepcidin levels between time-points when hepcidin levels were measured using ELISA.

The study has two principal limitations: the sample size was small and only fraction of the factors (e.g. cytokines) that are known to influence the levels of proteins involved in iron metabolism were measured. Thus, we were unable to elucidate fully the complex regulation of iron metabolism in hyperthyroid patients.

From a clinical point of view, knowledge of these dynamic changes and the underlying mechanisms is critical, allowing the clinician to estimate the impact of thyroid function abnormalities on these parameters, to consider other differential diagnoses and to prevent unnecessary investigations (e.g. for clarification of hyperferritinemia). The role of these acute phase mechanisms in GH in the clinical context should be further elucidated. As with cytokines[41] or TRAb-titers[42], hepcidin and ferritin levels could have a potential role as parameters for predicting relapse of GH after thyrostatic treatment. However, this topic has to be clarified in future studies.

In conclusion, our study results indicate that iron metabolism in patients with GH undergoes dynamic changes that are comparable to an acute phase reaction. Gender and smoking status had an impact on parameters of iron metabolism and thyroid hormones, in contrast to inflammatory parameters and cytokines which were unaffected by thyroid status. For the first time, we were able to demonstrate that hepcidin, a key regulator of human iron metabolism, is affected in Graves' hyperthyroidism, and that thyroid hormones had a direct effect on expression of this protein in hepatocytes.

Declaration of interest

There is no conflict of interest. The authors have nothing to disclose.

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Figure Legends

Fig. 1. Change in serum ferritin levels between baseline (T0) and follow-up measurements (T1).

Interaction model adjusted for sex and age at baseline (time between measurements and smoking status were also considered).

Fig. 2. Change in serum hepcidin$_{MS}$ levels between baseline (T0) and follow-up measurement (T1).

Interaction model adjusted for sex and age at baseline (time between measurements and smoking status were also considered).

Fig 3. Change in fT4 (A) and fT3 (B) between baseline (T0) and follow-up measurements (T1), showing the difference between smokers and non-smokers.

3 A: Test for interaction between time and smoking status (suggesting differences in the dynamics of the change according to smoking status): P = 0.011. ** P=0.016 (Wald test).

3 B: Test for interaction between time and smoking status (suggesting differences in the dynamics of the change according to smoking status): P = 0.004. ** P=0.006 (Wald test).

Fig. 4. Hepcidin mRNA expression in HepG2 cells stimulated with T3 versus control (Co).

** P<0.01.

Table 1. Thyroid parameters, mediators of iron metabolism and inflammatory markers.

Parameter [unit]	Reference range	n complete measure-ment pairs (T0&T1)	Baseline (T0)	Follow-up (T1)	P-value[1]
TSH [mU/l]	0.27-4.2	31	<0.01 (0.01-0.01) [0.01-0.02]	1.54 (0.59-2.74) [0.01-6.11]	<0.001
fT4 [pmol/l]	12.0-22.0	31	35.3 (26.7-51.9) [12.3-100.1]	13.8 (11.1-15.0) [7.8-22.0]	<0.001
fT3 [pmol/l]	3.1-6.8	31	13.8 (9.0-23.1) [4.3-30.8]	4.4 (4.1-5.0) [2.6-6.7]	<0.001
TRAb [U/l]	<1.8	31	6.2 (2.8-12.2)	1.5 (0.5-4.0)	<0.001
Plasma Iron [µmol/l)	F: 6.6-26 M: 11-28	31	16.8 (9.2–21.9)	19.1 (16.3–23.3)	0.143
Transferrin [g/l]	2.0-3.6	31	2.3 (2.1–2.5)	2.6 (2.4–2.8)	0.002
Transferrin Saturation [%]	16-45	31	30.3 (20.3–42.0)	31.8 (23.5–38.3)	0.7
Soluble Tf-receptor [mg/l]	F: 1.9-4.4 M: 2.2-5.0	31	2.4 (2.1–2.9)	2.7 (2.2–3.4)	0.138
Ferritin [µg/l]	F^2: 23-160 F^3: 30-400 M: 30-400	31	156.0 (63–262)	80.0 (38–141)	0.015
$Hepcidin_{MS}$ [nmol/l]	F^2: 0.4-9.2 F^3: 0.7-16.2 M: 1.1-15.6	25	9.0 (6.5–17.1)	5.9 (4.3–7.6)	0.011
$Hepcidin_{EL}$ [ng/ml]	4.69-300[4]	25	185 (108–277)	115 (63.75–178.5)	0.0533
hsCRP [mg/l)	<5mg	31	1.3 (0.4–4.6)	0.9 (0.3–3.1)	0.750
PCT [µg/l]	<0.1	31	0.04 (0.04–0.05)	0.04 (0.03–0.05)	0.163
IL-1β [pg/ml]	0.0-3.9	30	0.10 (0.00–0.25)	0.05 (0.00–0.48)	0.838

IL-6 [pg/ml]	0.0-3.1	30	2.00 (1.40–3.45)	1.45 (0.90–3.13)	0.248
TNF-α [pg/ml]	0.0-6.3	30	0.00 (0.00–0.45)	0.25 (0.00–0.60)	0.233

Values expressed as median (IQR)¸ ([range] thyroid function tests)
TSH: Thyroid-stimulating hormone
fT4: Free T4
fT3: Free T3
TRAb: Thyroid receptor antibodies
$Hepcidin_{MS}$: Hepcidin-25 determined by mass spectrometry
$Hepcidin_{MS}$: Hepcidin determined by ELISA
F: reference range for females
M: reference range for males

[1] Difference between baseline (T0) and follow-up (T1) by Wilcoxon signed-rank test
[2] Reference range for females 18-50 years
[3] Reference range for females >50 years
[4] No gender-/age-specific reference ranges available

Table 2. Multivariable regression analyses.

	Ferritin [µg/l]		$Hepcidin_{MS}$ [nmol/l]	
	univariable	**multivariable**	**univariable**	**multivariable**
Intercept	*222.55 [142.10; 303.00]*	*205.80 [24.49; 387.11]*	*11.42 [8.42; 14.42]*	*18.32 [11.32; 25.32]*
Age at baseline (per year increase)		*4.36 [0.67; 8.04]*		
Female sex		*-259.29 [-450.93; -67.65]*		*-9.07 [-16.57; -1.58]*
Smoker				
Time between T0 and T1				-1.50 [-3.16; 0.16]
Change from T0 to T1	*-107.39 [-155.83; -58.94]*	*-217.67 [-344.81; -90.52]*	*-4.86 [-7.40; -2.33]*	*-8.35 [-12.99; -3.71]*
Interaction of T1 and female sex[1]		*155.39 [24.97; 285.82]*		*6.57 [1.21; 11.92]*
Interaction of T1 and smoking[2]				

	fT3 [pmol/l]		fT4 [pmol/l]	
	univariable	**multivariable**	**univariable**	**multivariable**
Intercept	*16.09 [13.17; 19.02]*	*18.47 [14.29; 22.64]*	*41.55 [33.70; 49.39]*	*48.58 [37.47; 59.70]*
Age at baseline (per year increase)				
Female sex				
Smoker		*-6.14 [-11.11; -1.16]*		*-18.18 [-31.15; -5.21]*
Time between T0 and T1				
Change from T0 to T1	*-11.55 [-14.54; -8.56]*	*-14.05 [-18.36; -9.75]*	*-27.90 [-35.56; -20.25]*	*-34.89 [-45.85; -23.94]*
Interaction of T1 and female sex[1]				
Interaction of T1 and smoking[2]		*6.47 [1.48; 11.47]*		*18.06 [5.63; 30.49]*

Results [95% CI], printed in italic indicate $p < 0.05$
[1] Difference in change between sexes
[2] Difference in change between smokers and non-smokers

Figures

Figure 1

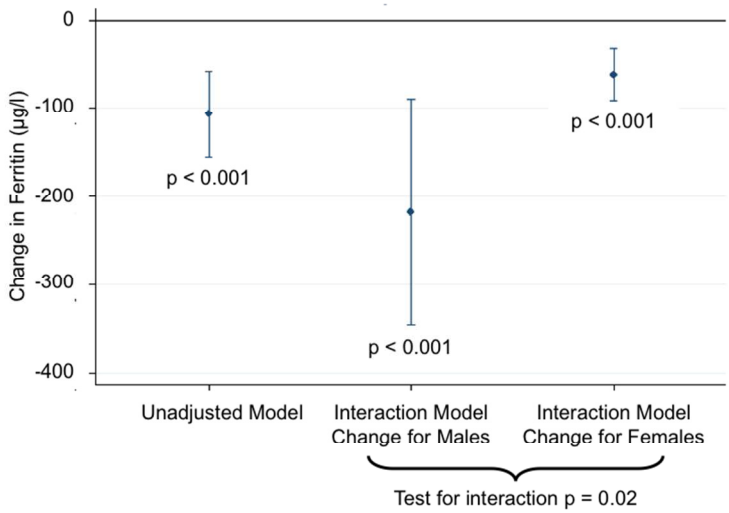


Figure 2

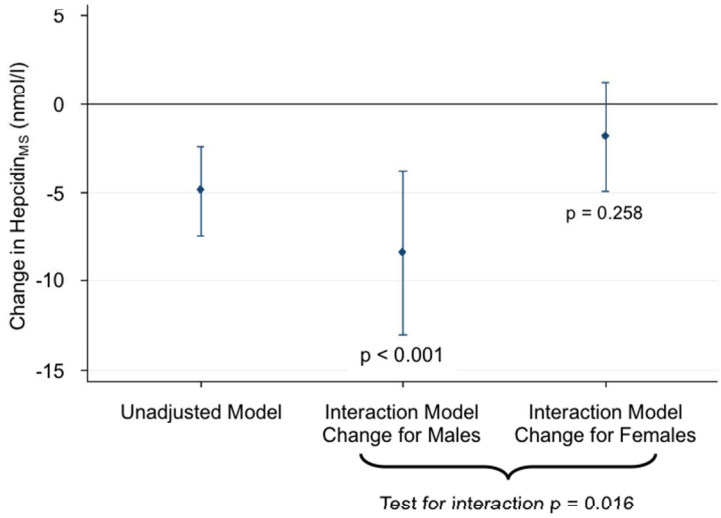
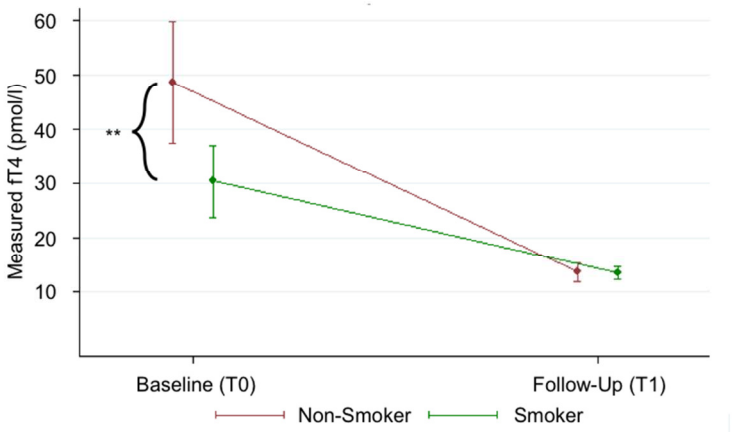


Figure 3a/3b


60
50
40
30
20
10
Measured fT4 (pmol/l)
**
Baseline (T0)
Follow-Up (T1)
Non-Smoker
Smoker

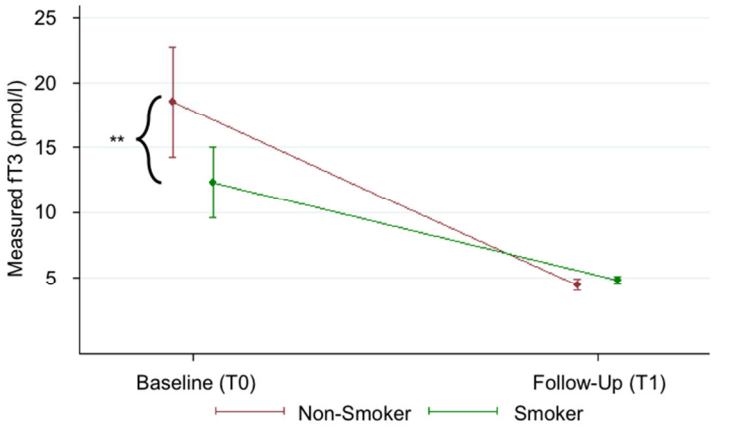

25
20
15
10
5
Measured fT3 (pmol/l)
**
Baseline (T0)
Follow-Up (T1)
Non-Smoker
Smoker

Figure 4